Effects of low gamma radiation from ^{60}Co on *in vitro* shoots of *Mentha arvensis* L.

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Abstract
Gamma irradiation is an effective mutagenic technique for inducing genetic variations and developing improved varieties of peppermint (Mentha arvensis L.), an important essential oil crop. This study investigated the effects of different gamma radiation doses (0, 2, 4, 6, and 10 Gy) on in vitro shoot cultures and field performance of peppermint. Irradiation at 6 Gy proved optimal, significantly enhancing shoot growth parameters like number of shoots, shoot height, number of leaves, and fresh weight compared to non-irradiated controls and higher doses during in vitro culture. When transplanted to the field, the 6 Gy irradiated peppermint plants exhibited superior vegetative growth, including higher plant height, more leaves and branches, and greater dry biomass accumulation at 30, 60, and 80 days after transplanting. Importantly, these plants had a 10% higher essential oil content along with remarkable 63% and 64% increases in the key aroma compounds menthol and menthone respectively, relative to non-irradiated controls.

The results highlight low-dose gamma irradiation as an effective mutation induction approach for peppermint improvement, simultaneously enhancing growth traits, essential oil yield, and oil quality with enriched menthol and menthone levels. The optimal 6 Gy dose identified provides valuable insights for mutation breeding aimed at genetic enhancement of peppermint and other essential oil crops.

Keywords: Mint, *Mentha arvensis* L., ^{60}Co radiation, gamma irradiation, essential oil, menthol, menthone.

Introduction

Peppermint (*Mentha arvensis* L.) is a species belonging to the Lamiaceae family, widely cultivated for the commercial value of its peppermint essential oil. This essential oil has been and continues to be a source of raw material for many industries such as food, cosmetics, and pharmaceuticals due to its antibacterial, analgesic, antioxidant, and deodorizing properties[14]. The content and chemical composition of the essential oil, in addition to growth yield, determine the quality of peppermint plants[5]. To achieve a higher menthol content, appropriate breeding measures must be taken. Some commonly used new variety breeding methods include irradiation, chemical mutagenesis, and hybridization[10]. Among these, gamma irradiation is one of the most effective techniques to induce genetic mutations and create new lines with desired characteristics.

In a 1996 field study in Bulgaria, several peppermint varieties underwent gamma irradiation with ^{60}Co (at doses of 0.2, 0.5, 1, 2, and 5 kR). Different cultivars were studied including cv Tundza, cv Zephir from *Mentha piperita* L., and cv Mentolna-14 from *Mentha arvensis* L. var. *piperascens Malinv*. Gamma irradiation enhanced the fresh green yield and root yield in both species, however, with different optimal doses for each. The 1000 R dose raised the amount of menthol in the oil and the yield of organic oil in the Tundza cultivar of *Mentha piperita* L.[17]. However, the total oil yield went down because the plant's oil content went down after irradiation.

In 2015, ^{60}Co irradiation doses were applied to *Mentha arvensis* L., but the plants were regenerated from callus tissue. The low-dose irradiated samples showed no morphological differences compared to the control plants, but they improved growth and development performance particularly the 6 Gy irradiated regenerated peppermint, which had 1.3 times higher essential oil content than the control lot[15]. The high-dose irradiated samples exhibited many morphological changes, some variants also showed better growth, development, and essential oil content than the control plants.

In 2021, a study tried to create better mutant types of *Mentha piperita* that had the shape and oil content of M. arvensis but the oil quality of M. piperita. Gamma-irradiation at different doses (10, 20, 30, 50, 70, 90, and 110 Gy) induced a series of variations in agronomic morphological traits, yield, and quality attributes. Superior mutants were selected and developed[11].

Recently, in 2024, scientists tested gamma doses of 10, 20, 30, 50, 70, 90, and 110 on the stems and seeds of the *Mentha piperita* genotype MPS-36. This genotype is high in menthofurans. A novel and promising mutant, MPS-36(S-3-3) was identified with distinct peppermint oil quality globally, with high menthol content (68-78%) and low menthofuran content (0.2-0.8%). They subsequently commercialized this mutant type[12].

Gamma irradiation has proven to be an effective technology over many decades for inducing genetic variations and creating new, improved quality lines in peppermint (*Mentha* spp.). However, the irradiation doses used and the plant parts subjected to irradiation varied across studies, leading to diverse results. In this study, radiation doses of 0, 2, 4, 6, and 10 Gy were applied to *in vitro* shoot samples to enhance yield and oil quality in peppermint (*Mentha arvensis* L.) in Vietnam. The samples with the best shoot development quality were further field-tested to evaluate growth, development, and the total essential oil, menthol, and menthone contents.

Material and Methods

Gamma irradiation on *in vitro* mint shoot samples: Peppermint plants (*Mentha arvensis* L.) were used to grow the *in vitro* peppermint shoot samples. The plants were grown in Murashige and Skoog (1962) medium[7,9] that had 3% sucrose, 0.01% myo-inositol, 0.01% amino acid, and 0.37% gelrite added to it. We performed gamma irradiation at doses of 2, 4, 6, 8, and 10 Gy when the peppermint shoot samples reached about 2 cm in height. The irradiated shoot samples were immediately transferred back to MS medium for further culture. Non-irradiated samples were used as a control. Growth parameters of the shoots, including shoot height (cm), number of shoots per clump, number of leaves per shoot, and shoot weight (mg), were monitored after 2-3 weeks.

Experimental planting in the field: We grew the *in vitro* plants irradiated at a dose of 6 Gy until they reached a height of 10 cm, and then transplanted them to the field. We also selected non-irradiated *in vitro* control plants at an equivalent height. The experiment was arranged in a completely randomized design with two treatments: irradiated plants and non-irradiated control plants. Each treatment was replicated three times, with nine plants per replicate. The growth and development of the control and irradiated plants were monitored over 3 stages at 30, 60, and 80 days after transplanting, with parameters including plant height (cm), number of leaves per plant, and number of branches per plant. The dry weight (g) of the plants was only calculated after harvesting on day 80.

Screening of essential oil, menthol, and menthone contents: After harvesting on day 80, the peppermint plants were air-dried at 30°C until a constant weight, then subjected to essential oil extraction by hydrodistillation. The extracted essential oil was stored at 4°C. The essential oil content (%) was calculated using the formula[16]:

$$\text{Oil content (g/100g)} = \frac{\text{Oil concentration}\left(\frac{\text{mg}}{\text{mL}}\right) \times \text{Oil volumn (mL)}}{\text{Dried sample weight (mg)}} \times 100$$

The menthol and menthone content in the peppermint essential oil were analyzed using a Hewlett-Packard HP5890 GC system coupled with a 5971 MSD mass spectrometer detector. The formula is as follows[15]:

$$\text{Menthol (or menthone) content (\%)} = \frac{\text{Menthol (or menthone) weight}}{\text{Oil weight}} \times 100$$

The measurements were repeated three times for each treatment.

Statistical analysis: The experimental data were analyzed for variance[8], and the means were compared using the LSD (Least Significant Difference) test. The statistical analyses were conducted using SAS[13], at 5% probability level.

Results and Discussion

The effects of gamma radiation on the shoot growth of peppermint plants: The *in vitro* peppermint shoots were irradiated to investigate the stimulatory effect on the growth and development of *in vitro* samples. The results showed that radiation doses from 2 Gy to 4 Gy and 6 Gy gradually increased the shoot differentiation ability, with the highest at the 6 Gy dose. However, at doses higher than 6 Gy (such as 8 Gy and 10 Gy), the growth parameters of peppermint shoots tended to decrease, indicating the adverse effects of excessive radiation doses (Table 1). Specifically, the number of shoots per clump tended to increase with increasing gamma radiation doses up to 6 Gy (23.2 shoots/clump), doubling compared to the control (10.6 shoots/clump), and then decreasing at higher doses. The 6 Gy dose also recorded the highest shoot height (7.0 cm) while the 10 Gy dose yielded the lowest shoot height (5.2 cm) (Figure 1).

The number of leaves per shoot and the fresh weight of shoots also gradually increased with radiation dose and reached the highest values at 6 Gy with 11.8 leaves/shoot and 557 mg/shoot, significantly higher than the control (10.4 leaves/shoot and 360 mg/shoot respectively).

Table 1
Effect of doses of gamma irradiation on shoot growth of *in vitro Mentha arvensis* L.

Doses (Gy)	Number of shoots per clump	Shoot height (cm)	Number of leaves per shoot	Shoot weight (mg)
0	10.6 ± 1.26^{d}	5.8 ± 0.95^{b}	10.4 ± 0.32^{b}	360 ± 1.00^{f}
2	15.2 ± 1.10^{c}	6.0 ± 0.30^{b}	10.4 ± 0.23^{b}	460 ± 1.30^{d}
4	17.6 ± 1.83^{b}	5.9 ± 0.36^{b}	10.5 ± 0.30^{b}	436 ± 0.86^{e}
6	$\mathbf{23.2 \pm 0.94^{a}}$	$\mathbf{7.0 \pm 0.20^{a}}$	$\mathbf{11.8 \pm 0.10^{a}}$	$\mathbf{557 \pm 1.07^{a}}$
8	15.0 ± 0.90^{c}	5.5 ± 0.49^{b}	9.4 ± 0.49^{c}	502 ± 1.89^{b}
10	13.1 ± 0.80^{c}	5.2 ± 0.30^{b}	9.9 ± 0.30^{bc}	476 ± 3.27^{c}
CV (%)	7.5	8.5	3.58	0.38

Note: In the same column, mean values followed by the different letters are statistically different ($P < 0.05$).

The coefficient of variation (CV%) of the monitored indicators was all low (below 10%). This showed that the collected data was highly accurate and reliable, with small fluctuations around the mean value.

The effects of gamma radiation on the plant growth in the field: The initial plants were selected to be equivalent in shoot height (10 cm), number of leaves, and number of branches. At all monitoring times (30, 60, and 80 days after planting), gamma irradiation at a dose of 6 Gy significantly stimulated the growth of peppermint (*Mentha arvensis* L.) compared to the non-irradiated control.

Specifically, after 30 days, the 6 Gy irradiated plants had a significantly higher average height (21.5 cm) than the control (18.8 cm). The number of leaves per plant (28.8 leaves/plant) and the number of branches (14.1 branches/plant) were also higher than the control (25.1 leaves/plant and 9.8 branches/plant) (Figure 2). After 60 days, the difference between the irradiated and control plants was even more pronounced. The 6 Gy irradiated plants had a height of 30.5 cm, 35.9 leaves per plant, and 23.0 branches per plant, which were 21.5%, 21.7%, and 21.7% higher than the control respectively.

At 80 days after planting, the 6 Gy irradiated plants still maintained their advantage in height (33.0 cm), number of leaves (46.6 leaves per plant), number of branches (31.4 branches per plant), and dry matter content (27.3 g), which were 15.2% higher than the control (Figure 3).

This demonstrates that gamma irradiation at a dose of 6 Gy had a prolonged and stimulatory effect on the growth in height, number of leaves, number of branches, and dry matter accumulation of peppermint throughout its growth and development. The differences in average height, number of leaves, and number of branches between the irradiated and control plants were statistically significant (P values < 0.05) at all monitoring times.

In 2015, Tran et al[15] also conducted a study irradiating the peppermint plant *Mentha arvensis* L. with both low irradiation doses (3, 4, 6, 8, 10 Gy) and high doses (30, 40, 50, 60, and 70 Gy). The plants were monitored at time points of 20, 40, and 60 days. To make the discussion comparable, we accounted for their and our research results at the same 60-day screening.

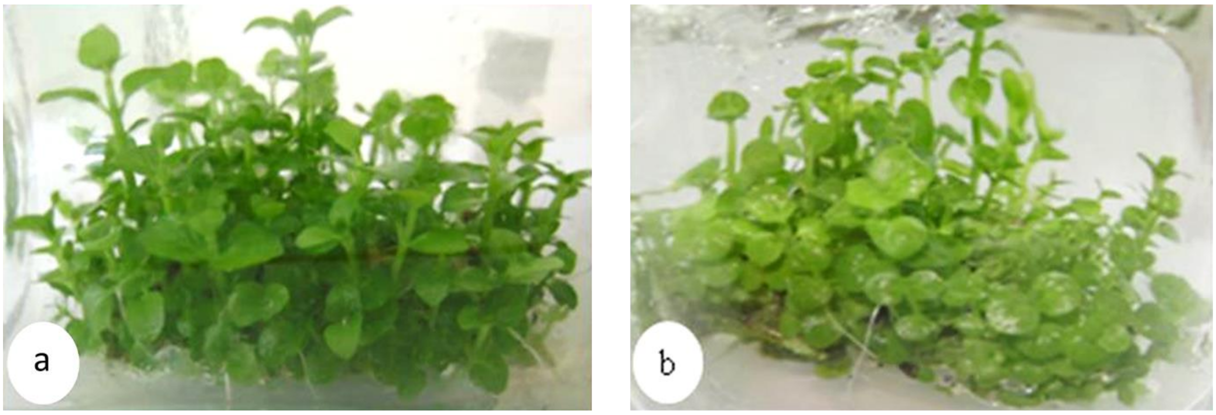

Figure 1: The growth of mint shoots irradiated with (a) 6 Gy and (b) control

Figure 2: The growth of mint plants irradiated with (a) 6 Gy and (b) control after 30 days of planting in the field.

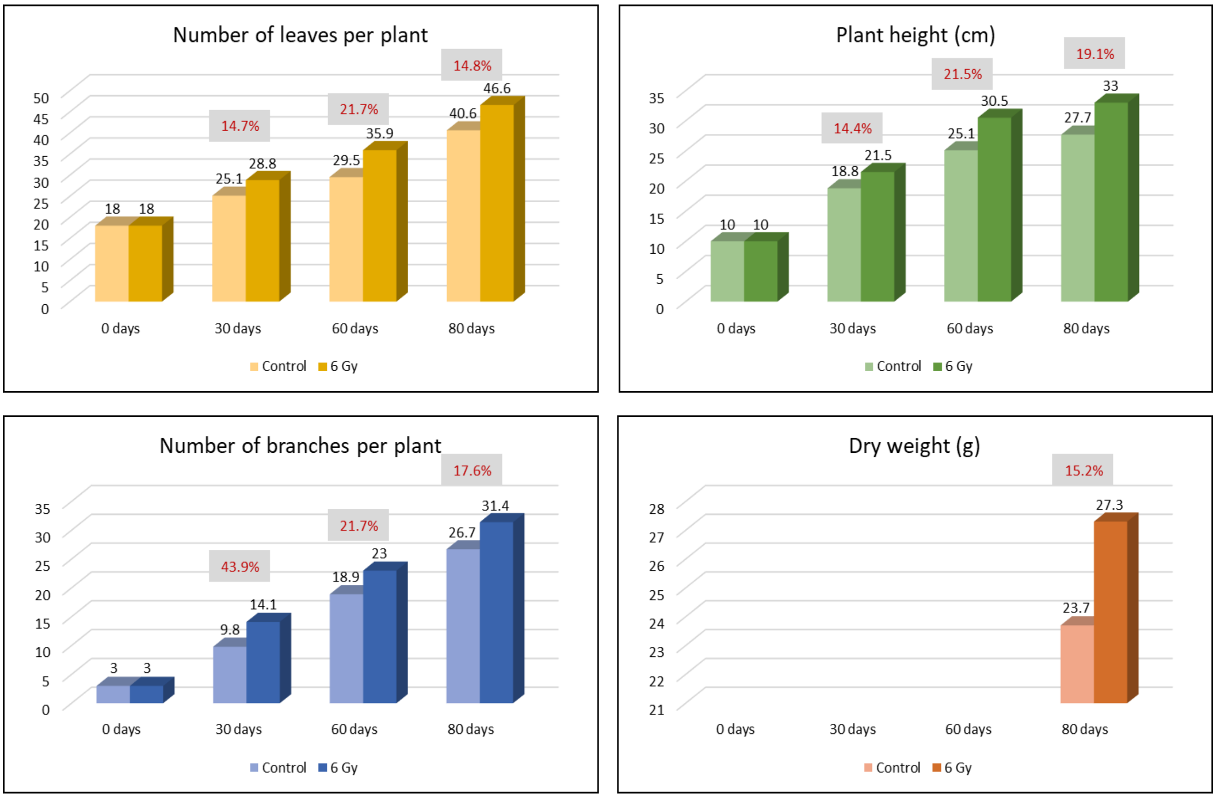

Figure 3: Effects of gamma irradiation on plant growth of *Mentha arvensis* L. in the field after 30, 60, and 80 days of monitoring.

Notes: The red values represent the increasing rate (%) of the irradiation samples in comparison with the control ones.

Among the low irradiation doses, the 6 Gy dose from the previous study[15] also showed the best plant growth as compared to lower doses (0, 3, 4 Gy) and higher doses (8, 10 Gy). The plant height reached 31.4 cm, and the number of branches per plant was 17.2 which was consistent with our results of 30.5 and 23 respectively. In the high-dose trials, 60 Gy induced the best plant height mutation (32.2-33.5 cm), with the highest number of branches per plant (20.4).

Compared to our low-dose irradiation results, these are not significantly different. Meanwhile, the high irradiation dose, although capable of producing some good individuals, often caused plant deformities at a higher rate, lower survival rates, and sometimes even inhibited the development of the cultured samples[1,4]. Thus, a higher irradiation dose is not necessarily a suitable condition for good plant growth and development. In contrast, low doses of gamma rays are more suitable for increasing the plant's resistance to the natural environment and reducing mortality due to sudden environmental changes in *in vitro* plants[2].

Additionally, the irradiated sample is another point of difference to note. Tran Minh et al[15] studied irradiated callus samples whereas our study applied radiation to shoot specimens. Both shoot irradiation and callus irradiation are common methods used in research and application to create new plant variants. Callus irradiation is easy to process and allows strong regeneration of new plants from irradiated cells[3]; however, since callus typically contains many cells and has high regeneration ability, irradiation can become unstable after irradiation, leading to the creation of undesirable variants.

In contrast, direct irradiation of plant shoots allows accurate and easy measurement of the radiation dose, as the radiation only directly affects the target cells[6]. Shoot irradiation can also create many new plant variants in a short time. However, shoot irradiation also has its own disadvantages, as shoots are more difficult to regenerate than callus, and the shoots are also more vulnerable, so high irradiation doses should not be used. Both methods have their own advantages and disadvantages and the choice between them often depends on the specific research objectives and characteristics of the crop plant.

Effects of gamma radiation on essential oil, menthol, and menthone contents in *Mentha arvensis* L. plant: The findings show that moderate gamma irradiation led to an increase in the amount of essential oil present. The peppermint plants exposed to 6 Gy (4.4 g/100 g dried sample) had 10% more essential oil than the radiation-free control plants (4 g/100 g dried sample). Notably, the menthol content (the main component of peppermint oil) in the 6 Gy irradiated plants was 78.3%, significantly higher (63% increase) compared to the control at only 48.03%. Similarly, the menthone content in the 6 Gy irradiated plants (6.02%) was also 64% higher than the control (3.67%) (Figure 4). All of these differences were statistically significant (P values < 0.05).

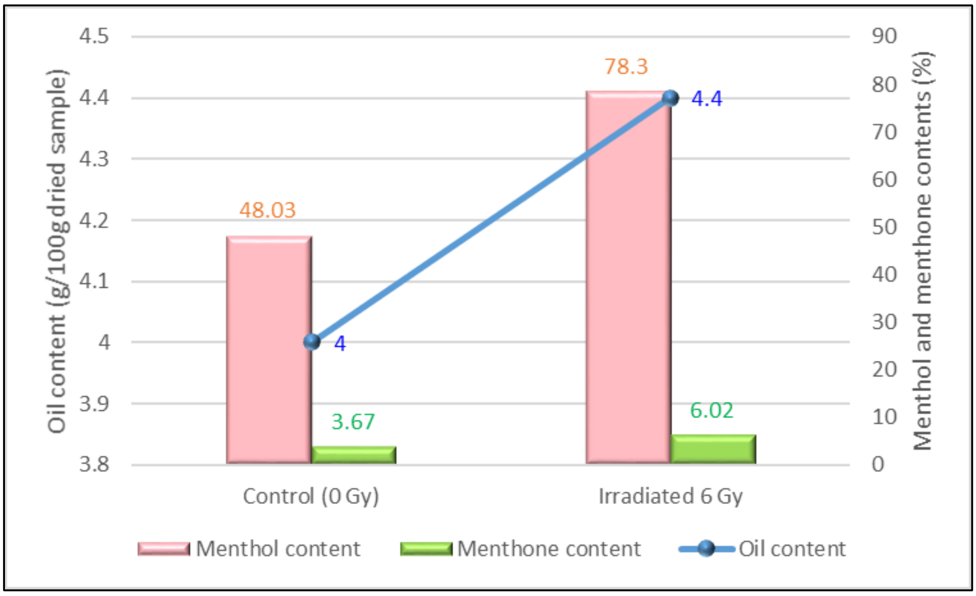

Figure 4: Effects of gamma irradiation on essential oil, menthol, and menthone contents of *Mentha arvensis* L. after 80 days planting in the field.

Hence, gamma rays not only increased the essential oil content but also markedly enhanced the biosynthesis and accumulation of menthol and menthone, important essential oils in peppermint, contributing to the increased value and economic efficiency of this mutant plant. Both menthol and menthone are major and important components of peppermint essential oil, with menthol typically accounting for a high proportion. The menthol content in the mutant plants in this study (78.3%) is entirely comparable to the menthol content (68-78%) of the commercialized *Mentha piperita* line MPS-36 (S-3-3), which was the best irradiated sample from the study by Prasad et al[12].

The study by Tran Minh et al[15] found that the best oil content at the low irradiation dose was 3.7 g/100g of dried samples at 6 Gy. For the high irradiation doses, 60 Gy also gave the best result, with an oil content ranging from 2.2 to 5.0 in different mutant lines. Our result of 4.4 g/100g of dried samples at 6 Gy is comparable and significantly higher than the average of the mutated samples from the previous study. Moreover, the menthol content in our sample increased dramatically from 48.3% to 78.3%, approaching the result of the best mutant line in the previous study (81.1%). This once again confirms that a suitable low irradiation dose still has the potential to create high-yielding mutants, depending on the sample's radiation sensitivity.

Conclusion

This study demonstrated the positive effects of low-dose gamma irradiation (6 Gy) on growth parameters, essential oil content, and essential oil composition in the peppermint plant *Mentha arvensis* L. In *in vitro* shoot cultures, the 6 Gy dose significantly enhanced shoot differentiation ability, with maximum values for number of shoots per clump, shoot height, number of leaves per shoot, and shoot fresh weight compared to non-irradiated controls and higher radiation doses.

When transplanted to the field, the 6 Gy irradiated peppermint plants exhibited superior growth and development compared to non-irradiated controls at all monitoring stages (30, 60, and 80 days). Importantly, gamma irradiation at 6 Gy led to a 10% increase in essential oil content and remarkable increases of 63% and 64% in the key components menthol and menthone, respectively compared to non-irradiated controls.

Overall, the results highlight the effectiveness of low-dose gamma irradiation as a promising technique to induce beneficial mutations and to develop new high-performing peppermint varieties with improved growth characteristics, higher essential oil yield, and enhanced essential oil quality, particularly enriched menthol and menthone contents. The successful identification of the 6 Gy treatment as the optimal dose provides valuable insights for future mutation breeding programs aimed at genetic improvement of peppermint and other essential oil crops through gamma irradiation.

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